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# The dynamics of Chinese variant porcine epidemic diarrhea virus production in Vero cells and intestines of 2-day old piglets

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ABSTRACT

A severe porcine epidemic diarrhea (PED) epizootic has been affecting pigs of all ages that are characterized by high mortality among suckling piglets in China since late 2010, causing significant economic losses. Obtaining a current-circulating PEDV variant isolate that can grow efficiently in cell culture is prerequisite for the development of efficient vaccines. In this study, PEDV strain HN1303 was isolated successfully on Vero cells with supplemental trypsin, and the isolate has been serially propagated in cell culture for over 95 passages. The infectious titers of the virus during the first 10 passages ranged from $10^{2.6}$ to $10^{5.8}$ 50% tissue culture infective doses ($TCID_{50}$)/ml, and the titers of 20–95 passages ranged from $10^{6.2}$ to $10^{8.0}$ $TCID_{50}$/ml. The growth curve of Vero cell-adapted HN1303 in cell culture was determined, and dynamics of virus production was confirmed by immunoperoxidase monolayer assay (IPMA). Sequence and phylogenetic analysis based on spike gene indicate that the HN1303 strain belongs to genotype IIa. In addition, the fourth passage cell-culture HN1303 was subjected to 2-day old piglets. All piglets orally inoculated developed severe watery diarrhea and vomiting within 24 hours post-inoculation (hpi) and died within 72 hpi. The results of animal experiments reveal that this strain is highly pathogenic to 2-day old piglets.



## 1. Introduction

Porcine epidemic diarrhea virus (PEDV), an agent causing a serious and highly contagious swine enteric disease, is an enveloped, single-stranded, positive-sense RNA virus belonging to the order *Nidovirale*, the family *Coronaviridae*, subfamily *Coronavirinae*, and genus *Alphacoronavirus* (International Committee on Taxonomy of Viruses, 2012). This disease was first observed in England in 1971, and subsequently reported in many swine-producing countries in Europe and Asia (Pensaert and de Bouck, 1978; Song and Park, 2012). Severe PED epizootics have been affecting pigs of all ages characterized by high morbidity and mortality rates among suckling piglets in China since late 2010, resulting in huge economic losses, although most herds previously had received CV777-based inactivated or attenuated vaccines (Sun et al., 2012; Wang et al., 2013).

Developing efficient live attenuated or inactivated vaccines against PEDV remains the best solution for prevention and control of the disease. Obtaining an epidemic PEDV isolate that can grow efficiently in cell culture is critical for the development of vaccines. There are previous reports of successful propagation of several classical PEDV strains using Vero cells and trypsin in the medium, such as CV777, KPEDV-9, and 83P-5 (Hofmann and Wyler, 1988; Kusanagi et al., 1992; Kweon et al., 1999). Recently, Oka et al. (2014) and Chen et al. (2014) reported successful serially propagation of the highly virulent US PEDV strains in cell cultures for over 30 passages.

In this study, we attempted to isolate and propagate PEDV from four PEDV-positive intestinal samples using Vero cells supplemented with trypsin, and successfully isolated one PEDV strain designated as HN1303. The PEDV HN1303 was successfully serially passaged for over 95 passages, and the growth and titers of which in cell culture were characterized. Furthermore, the 4th passage

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of PEDV HN1303 was used for experimental infection of 2-day old piglets to determine its virulence. Immunochemistry staining was applied to different parts of small intestines at different time points after infection to understand the progression of infection.

## 2. Materials and methods

### 2.1. Clinical samples

In January 2013, four intestinal samples were obtained from 3-day old piglets with diarrhea from a farm in Henan Province, China. These samples were tested positive by a PEDV M gene-based RT-PCR at National Research Center for Veterinary Medicine, and were selected for virus isolation attempts. Each small-intestine tissue was homogenated in sterile phosphate-buffered saline (PBS), and was vortexed briefly followed by centrifugation at 8000 × *g* for 10 min at 4 °C. The supernatant went through 0.22 μm Millipore filters, and was used as an inoculum for virus isolation.

### 2.2. Virus isolation, serial propagation, and titration

Virus isolation of PEDV was attempted on Vero cells (ATCC CCL-81) as previously described (Hofmann and Wyler, 1988) with some modifications. Briefly, Vero cells were grown in Dulbecco Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 5% fetal bovine serum (Hyclone, USA) and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin). Confluent Vero cell monolayers were used for virus inoculation. Prior to inoculation, growth medium was removed and the monolayers of cells were washed with PBS twice in T25 flask. Then 0.5 ml of inoculum and 0.5 ml maintenance medium was added. Maintenance medium was consisted of DMEM supplemented with antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin), 0.3% tryptose phosphate broth (Sigma, USA), 0.02% yeast extract (Sigma, USA), and 10 μg/ml trypsin 1:250 (BD, USA). After adsorption at 37 °C for 60 min, 4 ml maintenance medium were added to each flask. The cultures were incubated at 37 °C with 5% $CO_2$, and examined for cytopathic effect (CPE) twice daily. Eighty percent of maintenance medium was changed daily. When CPE appeared in more than 80% of cells (~5 days after inoculation), the flasks were subjected to freeze-thaw twice. The cells and supernatants were mixed and stored at −70 °C. These samples were used as seed stocks for the next passage. If no CPE was observed within 5 days, 4 blind passages were performed using inoculated culture fluid as inoculum.

Virus titration was performed in 96-well plates with 10-fold serial dilutions performed in eight replicates per dilution. After 5 days of inoculation, the virus titers were determined according to the Reed and Muench method (Reed and Muench, 1938) and expressed as the 50% tissue culture infective dose ($TCID_{50}$)/ml.

### 2.3. Determination of virus growth curve

To determine virus growth curve, 750 $TCID_{50}$ 3rd and 79th passages of PEDV HN1303 were inoculated onto cell monolayers in 35 mm dishes. After adsorption at 37 °C for 60 min, the cells were fed with maintenance medium, and incubated at 37 °C with 5% $CO_2$. At 6, 9, 12, 15, 18, 21, 24, and 27 hours post-inoculation (hpi), cells were collected for virus titration. Virus titration at different time points was performed in triplicates.

### 2.4. IPMA

PEDV-infected Vero cells in 35 mm dishes were fixed with 80% acetone for 20 min, and then washed by PBS, air dried, and incubated with a 1:500 dilution mouse monoclonal antibody 3F12 (MEDIAN, Korea) specifically against PEDV spike in a humidity chamber at 37 °C for 40 min. After three washes with PBS, the cells were incubated for 30 min at 37 °C with HRP-labeled goat anti-mouse IgG (Biomedical Technologies Inc., USA) diluted 1:500 in PBS. The dishes were washed 3 times with PBS, followed by incubation for 4–5 min at room temperature in diaminobenzidine solution (ZSGB-BIO, Beijing, China). Cell staining was examined by using a light microscope.

### 2.5. S gene sequencing and phylogenetic analysis

The full-length spike (S) gene sequence of PEDV strain HN1303 was determined by the traditional Sanger method. Viral RNA extraction was performed with 200 μl HN1303 isolation at the passage 4 by using a viral nucleic acid extraction kit (Geneaid Biotech Ltd., Taiwan, China) following the instructions of the manufacturers. cDNA for the S gene was generated by EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) using a reverse primer PEDV-N (5′-GGCATAGAGAGATAATGGCA-3′). The S gene was amplified in three fragments using 2× TransStart FastPfu PCR SuperMix (TransGen Biotech, Beijing, China). Primers used were previously described by Park et al. (2007). The three fragments were amplified under same program of 3 min at 94 °C, 35 cycles of 20 s at 94 °C, 15 s at 58 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min. Purified PCR products were cloned using pEASY-Blunt Cloning Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Recombinant DNA clones were sequenced by Invitrogen of Life Technologies (Shanghai, China). Sequence obtained was aligned with reference sequences in the GenBank using ClustalX version 1.81, and the default setting with manual adjustment. Neighbor-joining tree based on the S gene was generated using the software PHYLIP version 3.67 (Retief, 2000). The reliability of branches in the tree was assessed by bootstrap analysis using 1000 replicates. The degree of similarity among sequences was determined using MegAlign version 7.0 (a tool in the software DNAStar). The full-length S gene nucleotide sequence of PEDV strain HN1303 in this study was deposited in the GenBank database under accession number KR080551.

### 2.6. Experimental infection of 2-day old piglets

Two experiments were performed and approved by the Animal Care and Ethics Committee of China National Research Center for Veterinary Medicine. Colostrum-deprived piglets used in both experiments were obtained from sows free of PEDV, porcine transmissible gastroenteritis virus, group A rotavirus, porcine circovirus 2, porcine reproductive and respiratory syndrome virus, and pseudorabies virus. In the first experiment, to determine the virulence of the PEDV strain HN1303, seven 2-day old piglets were randomly allocated into a PEDV-infected group ($n = 5$) or a negative control group ($n = 2$). Piglets in infected group were inoculated orally 2 ml ($10^{5.2}$ $TCID_{50}$/ml) virus of HN1303 at passage 4. Piglets in negative control group were similarly treated with 2 ml virus-free cell culture media. All 5 infected piglets exhibited severe diarrhea and vomiting, and died within 70 hpi.

To understand the progression of PEDV infection, we carried out the second experiment. Nine 2-day old piglets were randomly allocated into an infected group (pig number 1–7, $n = 7$) or a negative control group (pig number 8–9, $n = 2$), and were administered by the same dose and route as in the first experiment. Clinical signs were monitored continuously. In infected groups, pig7 and pig6 were euthanized at 8 and 12 hpi individually before the appearance of clinical symptoms. All the other piglets in infected group showed the clinical symptoms between 12 and 21 hpi and were euthanized at 8 (pig3), 17 (pig1), 27 (pig5), 35 (pig2), and 50 (pig4) hours post-appearance of clinical symptoms (hpacs) of each individual piglets. Pig8 and pig9 in negative control group were euthanized at 24 h and